Correlated responses of energy metabolism, development and reproduction to evolution for increasing heat tolerant in *Drosophila subobscura*.

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Abstract

1. Adaptation to increasing environmental temperatures implies an increase of the upper thermal limit (CTmax), but also an enhanced capacity of the organisms to perform fundamental biological functions at high temperatures.

2. Evolution of stress resistance is companied with a metabolic depression, which allows to organisms have reduced maintaining costs and allocate the energy surplus to energy demanding resistance mechanisms and reproduction.

3. Heat tolerance evolution depends on the heat intensity selection and it could have different consequences on the evolution of correlated traits such as energy metabolism and fitness-related traits. Thus, we evaluated the correlated responses of metabolic rate, metabolism-related enzymes (G6P branchpoint enzymes), reproductive and preadult survival in control and selected lines for increasing heat tolerance in *Drosophila subobscura*.

4. We did not find a correlated response of metabolic rate associated with heat tolerance evolution, but we detected a reduced hexokinase activity in the slow-ramping selected lines. On the other hand, we found an increase in fecundity in the fast- and slow-ramping selected lines, but an increase in egg-to-adult viability was only found in the slow-ramping selected lines.

5. Our results show that the evolution of heat tolerance does not imply an increase in energy demand neither a tradeoff with reproduction and preadult survival, which suggests that heat tolerance evolution induces correlated response to increase the performance at high temperatures. Therefore, spatial and temporal variability of thermal stress intensity should be taking into account in future studies if we want to understand and predict the adaptive response to ongoing and future climatic conditions.

Introduction

Environmental thermal stress involves strong selective pressures that can led to important evolutionary changes depending on the presence of genetic variation in natural populations (Umina et al, 2005; Crozier & Hutchings, 2014; Chick et al, 2020). The pervasive effects of the temperatures on ectothermic organisms cause that fitness, abundance and distribution of their populations are susceptible to the current and future increase of temperature (Deutsch et al, 2008; Angilletta, 2009; Chen et al, 2011). Therefore, it is crucial to know and understand the capacity of ectothermic organisms to withstand the global warming.

The ability to tolerate higher temperatures implies that together with an increase of the upper thermal limit, organisms must exhibit an array of responses of at different levels of biological organization, allowing them to maintain their biological performance at high temperatures (Huey & Kingsolver, 1993; Pörtner et al, 2006; Mesas et al, 2021; McGaughran et al, 2021).
Among these responses, metabolic depression, defined as a reduction of the energy expenditure associated to maintenance of organisms, is considered an important mechanism associated with resistance to different types of environmental stress (Guppy & Withers, 1999; Storey & Storey, 2004). Plastic and evolutionary decreases of energy metabolism should have important ecological and evolutionary implications in stressful conditions because it allows to organisms reduce their minimum energetic requirements and redistribute the energy surplus to biological functions close to fitness (Atkinson, 1977; Hochachka & Somero, 2002, Storey & Storey, 2004). For example, a reduced standard metabolic rate (SMR) has evolved by natural selection in the garden snail (Artacho & Nespolo, 2009), whereas evolutionary responses increasing tolerance to desiccation, starvation, hypoxia, and high temperatures have been accompanied by a reduction in metabolic rate (Hoffmann & Parson, 1989; Padfield et al, 2016; Regan et al, 2017; Brown et al, 2019).

In ectotherms, metabolism increases with environmental temperatures, inducing high metabolic costs in individuals exposed to heat stress as expected by global warming (Lighton & Turner, 2004; Huey & Kingsolver, 2019). Therefore, metabolic depression could be an important strategy to prevent that metabolism exceeds the physiological limits and energy expenditure, which has adversely effects on fitness under extreme heat conditions (Marshall & McQuaid, 2011; González-Tokman et al, 2020; Pilakouta et al, 2020). This metabolic response to high temperature could partially explain the counter gradient variation proposed in metabolic cold adaptation hypothesis (Addo-Bediako, 2002; Gaston et al, 2009), which establishes that ectotherm species from temperate habitats have lower metabolic rates than those living in colder environments (Addo-Bediako et al, 2002; Sylvestre et al, 2007; Schaefer & Walters 2010; Bruning et al, 2013; Sinnatamby et al, 2015; Pilakouta et al, 2020). On other hand, experimental populations exposed to warm environmental conditions during several generations showed an evolutionary increase of their thermal tolerance accompanied with a reduced metabolic rate (Padfield et al, 2016, but see Mallard et al, 2018). However, heat tolerance evolution and the correlated responses of associated traits depend on the intensity of heat stress selection (Santos et al 2012; Mesas et al, 2021). For instance, Mesas et al (2021) found evolutionary responses of heat tolerance regardless of intensity of heat selection, which did have an effect on the evolutionary response of the thermal performance in *Drosophila subobscura* (Mesas et al, 2021). The effects of thermal selection intensity on thermal-related traits were previously predicted by Santos et al (2012) whom, using mathematical simulation of artificial selection experiments, found a decrease of metabolic rate only when selection for increasing heat tolerance was performed using slow-ramping protocols. This correlated response between heat tolerance and metabolic rate can be explained because flies with low metabolic rate can withstand longer exposure in slow-ramping assays, resulting in higher thermal tolerance (Rezende et al, 2011; Santos et al, 2012).

Energy metabolism is emergent property of interactions between different cellular processes and metabolic pathways (Berrigan & Hoang, 1999; Montooth et al, 2003; Brown et al, 2004; Matoo et al, 2019). Then, exposure to different heat stress intensities should results into thermal dependence of metabolic rates and flux of metabolic pathways. For example, several genes associated with the glucose-6-phosphate (G6P) branchpoint, which has an important role in the storage and use of molecules as glucose, glycogen, triglycerides and ATP, have shown clinal variation in their allelic frequencies along latitude (Oakeshott et al 1983; Duvernell & Eanes, 2000; Verrelli & Eanes, 2001; Rank & Dahlhoff, 2002, Sezgin et al, 2004). These findings suggest that the evolution of functional and structural properties of the enzymes associated with energy metabolism are sensitive to environmental temperatures. Moreover, different haplotypes associated with G6P branchpoint can differentially influence the energy flux in a particular metabolic pathway (Eanes & Hey, 1986; Zera, 1987; Verrelli & Eanes, 2001), but also affect the flux in parallel metabolic pathways, facilitating correlated responses among fitness-related traits under stressful conditions (Zera & Zhao, 2003a). For example, evolution of stress resistance was associated with changes in metabolic rate, fecundity, and development in *D. melanogaster* (Hoffmann & Parson, 1989; Brown et al, 2019). On the other hand, evolution under warm conditions resulted in a down-regulation of metabolic pathways (e.g., genes associated with Krebs cycle, oxidative phosphorylation, and glycolysis enzymes) and an increase in fertility in *D. simulans* (Mallard et al, 2018). Notably, reproductive and developmental traits have been negatively associated with heat stress in in ectotherms (Morrison & Hero, 2003; Qu et al, 2011; Hamby et al, 2016; Porcelli et al, 2017), which should suggest that heat tolerance evolution involves metabolic readjustments that impact on energy-demanding traits such as fitness-related traits (Hochachka & Somero, 2002; Harshman & Zera, 2007; Tomanek & Zuzow, 2010). In this work, we evaluated the evolutionary change on energy metabolism and life history traits in response to artificial selection for increasing heat tolerance in *D. subobscura*, using two different

thermal selection protocols to measure heat tolerance: slow and fast ramping protocols, which involve different heat intensity selection regimens. We hypothesized that increased heat tolerance evolves associated with a reduction of energy metabolism, especially if thermal selection is performed using slow-ramping protocols in comparison to fast-ramping protocols. Additionally, we proposed that the metabolic reduction will decrease the maintenance energy requirements and generate an energy surplus, which should positively affect to reproductive and developmental traits. To evaluate these hypotheses, we measured the routine metabolic rate (RMR) and the activity of enzymes associated with the G6P branchpoint as proxies of the energy metabolism in experimental populations of *D. subobscura* previously selected for increasing heat tolerance (Mesas et al, 2021). We measured the activity of these enzymes in flies exposed to two different thermal conditions (non-stress: 21 ºC, and stress: 32 ºC) to evaluate if heat stress could impact on the enzymatic responses of selected lines. Additionally, we evaluated the early fecundity and egg-adult viability the evolved population to determine effect of heat tolerance evolution on life-history traits of *D. subobscura*.

Methodology

Heat knockdown temperature selection

A mass-bred population was established from the offspring of 100 isofemale lines derived of inseminated females collected in Valdivia, Chile (39.8 ºS; 73.2 ºW). These flies were maintained as a large outbred population, at 21 ºC (12:12 light:dark cycle) and feed with David's killed-yeast *Drosophila* medium. After 6 generations, this population was split into 12 experimental lines, which were assigned in triplicate to the different artificial selection protocols and their respective controls: fast-ramping control, fast-ramping selected, slow-ramping control, and slow-ramping selected lines. See details of the experimental evolution protocol in Mesas et al (2021).

For each replicated lines, 160 four-days old virgin females were randomly mated with two no-related males from the same replicated line. After two days, males were discarded, and vials were checked for positive oviposition. Then, 120 adult females were individually placed in a capped 5-mL glass vial, which was attached to two racks, each one with capacity to attach 60-capped vials. Racks were immersed in a water tank with an initial temperature of 28 ºC, which was controlled by a heating unit (Model ED, Julabo Labortechnik, Seelbach, Germany). After an equilibration time of 10 min, temperature was increased with a rate of 0.08 ºC min^{-1} for the flies belong to the slow-ramping selection protocol, or 0.4 ºC min^{-1} for the flies belong to the fast-ramping selection protocol. Each assay was photographed every 3 s with a high-resolution camera (D5100, Nikon, Tokyo, Japan). All photos for each assay were visualized to score the knockdown temperature for each fly, which was defined as the temperature at which each fly ceased to move. Flies were ranked according to their knockdown temperatures and we selected vials containing offspring of the 40 most tolerant flies (upper 30% of each assay) to found the next generation. For the fast-control and slow-control lines, the knockdown temperature was measured as mentioned above but offspring to found the next generation were randomly chosen.

Artificial selection for heat tolerance was performed for 16 generations, after which flies were dumped in acrylic cages and maintained without selection at 21ºC (12:12 light-dark cycle) until the measurement of metabolic and life-history traits. For logistic reasons, we used the fast -ramping control experimental line as control line for statistical comparisons.

Metabolic rate

The routine metabolic rate (RMR) was measured as the amount of CO_2 produced by individuals four days-old virgin females from the 26th generation. RMR was measured in 15 females from each experimental lines, reaching a total of 135 flies (3 thermal selection protocols x 3 replicated lines x

15 flies). Before metabolic measurements, flies were ice-cold anesthetized and weighted using a microbalance (MXA5, Radwag, Czech Republic). Then, flies were individually placed in a metabolic chamber, which were made of 2 cm of a Bev-A-Line tube and ending connectors glued with a cotton mesh to avoid that flies escape form the chamber. Metabolic chambers were connected to an eight-channel multiplexer system (RM8, Sable Systems International, NV, USA), with seven chamber containing individual flies and one empty chamber used for baseline. Metabolic chambers were placed in dark conditions within a thermal cabinet (PC-1, Sable Systems International, NV, USA) at a temperature of 21 ºC, which was controlled by a temperature controller (PELT-5, Sable Systems International, NV, USA). CO2-free air was pumped into chambers at a flow of 15 mL/min (Sierra MFC-2, Sable Systems International, NV, USA) and after 30 min of equilibration, CO2 production was sequentially measured in each chamber for 15 min using an infrared CO_2 analyzer with a resolution of 1 ppm (Li-6251, LI-COR Bioscience, NE, USA). Measurements of CO_2 were recorded, transformed to metabolic rate (µl CO_2/h), and analyzed using the software Expedata (Sable Systems International, NV, USA).

Enzyme activity

The catabolic activities of enzymes from the G6P branchpoint were measured in four days-old females from the 27th generation through the appearance of NADPH in kinetic assays. we evaluated the activity of hexokinase (HEX: E.C. 2.7.1.1) involved in glucose metabolism, phosphoglucomutase (PGM; E.C. 5.4.2.2) involved in glycogen metabolism, phosphoglucose isomerase (PGI; E.C. 5.3.1.9) involved in ATP metabolism; and glucose-6-phosphate dehydrogenase (G6PD; E.C. 1.1.1.49) involved in lipids metabolism.

From each replicated line, 100 females were collected and pooled in groups of 10 flies each. We measured the enzyme activity of the G6P branchpoint in flies exposed to two thermal

conditions: thermal-stressed flies (five replicates were exposed to 32 ºC per 1 h and then, flies were allowed to recover at 21 ºC for 2 h); and non-stressed flies (five replicates were maintained at 21 ºC for 3 h). Then, flies were quickly frozen in liquid nitrogen, homogenized in 1 ml of homogenization buffer (0.01 M KH_2PO_4, 179 1.0 mM EDTA, pH 7.4), and centrifuged at 2,000 rpm for 2 min at 4 ºC. The supernatant was aliquoted and maintained at -80 ºC until enzymatic assays. The buffers for each assayed enzyme were as follow. ***HEX:*** 20 mM TrisHCl, 0.5 mM NADP, 0.2 mM $MgCl_2$, 0.36 mM ATP, 5 mM D-glucose, 0.23 units/mL G6PD, pH 8.0. ***PGI:*** 20 mM TrisHCl, 0.28 mM NADP, 3 mM fructose-6-phosphate, 1.37 units/mL G6PD, pH 8.0. ***PGM:*** 20 mM TrisHCl, 0.5 mM NADP, 1 mM $MgCl_2$, 0.83 mM glucose-1-phosphate, 3.1 units/mL G6PD, pH 8.0. ***G6PD:*** 20 mM TrisHCl, 0.2 mM NADP, 18.8 mM $MgCl_2$, 3.5 mM glucose-6-phosphate, pH 8.0. Enzymatic assays were performed adding 25 μL extracts of flies with a protein concentration of 3500 μg/mL and 200 μL of respectively assay buffer in ultraviolet plates with flat bottom wells. Immediately, the optical density (OD) to 340 nm was measured with multiple reads per well in a microplate reader (Infinite 200 Pro, Tecan) previously heated to 29 ºC. The OD was measured each 25 s during 10 min to HEX and PGI, every 10 s during 10 minutes to PGM, and every 45 s during 15 min to G6PD. Enzymatic activities were estimated by triplicated and mean OD was analyzed. Blank wells containing buffer assays and double-distilled water (instead fly extracts) were measured to establish the basal optical density of each reaction. Finally, the enzymatic activity was calculated as increment rate of optical density through assay (ΔOD/s).

Early fecundity and egg-to-adult viability

Early fecundity was measured in fly females from 26th generation. For each replicated line, 10 virgin females were individually placed in vials with David's killed-yeast *Drosophila* medium together to two unrelated males from the same replicated line. After 24 h, females were transferred to new vials and

the number of oviposited eggs in each vial were daily counted using a stereomicroscope. This procedure was repeated every 24 h for eight days to obtain the accumulated fecundity for each female. Additionally, we measured the egg-adult viability as follow. Vials containing eggs oviposited by five days-old females (age of maximal activity of oviposition in *D. subobscura*) were retained to collect eggs. For each vial, 20 eggs were randomly transferred to a new vial, which were maintained to 21 ºC until flies will hatch.

Statistical analysis

Normality and homoscedasticity were tested for all variables, and only the enzyme activity of HEX was squared-root transformed to meet the parametric assumptions. Then, a mixed lineal model (hereafter 'full model') was used to evaluate the effects of thermal selection (fixed effect) and replicated lines nested within thermal selection (random effect) on body mass (eq. 1) and RMR (including body mass as covariate) (eq. 2):

$$Mb_{ijk} = \mu + Selection_i + Replicate(Selection)_{ij} + e_{ijk} \qquad \text{(eq. 1)}$$

$$RMR_{ijk} = \mu + Selection_i * Mb_{ijk} + Replicate(Selection)_{ij} + e_{ijk} \qquad \text{(eq. 2)}$$

For the enzyme activities, the full model included the interaction between the thermal exposure and thermal selection as fixed effects (eq. 3):

$$Activity_{ijkl} = \mu + Selection_i * Exposure_j + Replicate(Selection)_{ik} + e_{ijkl} \qquad \text{(eq. 3)}$$

Early fecundity was analyzed using a generalized lineal model with a Poisson distribution with thermal selection, oviposition days and their interaction were considered as fixed effects, while

replicated lines were nested within thermal selection were considered as a random effect (eq. 4). As fecundity was measured for each female along 8 days, females' identity (ID) was included in the model to accomplish for the repeated-measures design:

$$\text{Fecundity}_{ijkl} = \mu + \text{Selection}_i * \text{Day}_j + \text{Replicate(Selection)}_{ik} + \text{ID}_l + e_{ijkl} \qquad \text{(eq. 4)}$$

Finally, egg-to-adult viability was estimated as the proportion of emerging flies per vial, considering that 20 eggs were placed in a vial. This proportion was analyzed using a generalized lineal model with a binomial distribution with thermal selection as fixed effect, and replicated lines were nested within thermal selection were considered as a random effect (eq. 5):

$$\text{Proportion}_{ijk} = \mu + \text{Selection}_i + \text{Replicate(Selection)}_{ij} + e_{ijk} \qquad \text{(eq. 5)}$$

All statistical analysis was performed in R (R Core Team, 2021) using the function *lmer* and *glmer* of the *lme4* package for the generalized mixed model (Bates et al, 2014). Post-hoc analysis were performed using a Tukey-adjusted method using the *emmeans* package (Lenth, 2021).

Results

We found that knockdown temperature was significantly higher in fast-ramping selected lines compared to fast-ramping control lines ($\text{mean}_{\text{fast-ramping}} \pm \text{SD} = 37.71 \pm 0.68$ ºC and $\text{mean}_{\text{fast-control}} \pm \text{SD} = 37.23 \pm 0.79$ ºC; $F_{1,4} = 32.0$, $P = 0.005$), and knockdown temperature in slow-ramping selected lines was significantly higher than in slow-ramping control lines ($\text{mean}_{\text{slow-ramping}} \pm \text{SD} = 35.48 \pm 0.66$ ºC and $\text{mean}_{\text{slow-control}} \pm \text{SD} = 34.97 \pm 0.72$ ºC; $F_{1,4} = 41.7$, $P = 0.003$). The findings of the thermal selection experiments are detailed in Mesas et al (2021).

RMR and body mass

Body mass was not significantly different between the control and selected lines (χ^2_2 = 0.46, P = 0.79) and it did not differ between replicated lines (χ^2_1 = 0.03, P = 0.86). On the other hand, as expected, RMR and body mass were significantly correlated (r = 0.29, t_{132} = 3.48, P = 0.0007). Then, body mass was included as covariate in the mixed linear model, which showed that RMR did not differ between thermal selection regimens (χ^2_2 = 0.28, P = 0.87; Fig. 1) and neither between the replicated lines (χ^2_1 = 0, P = 1).

Enzyme activity

For HEX activity (Fig. 2A), we found a significant effect of the thermal selection regimens (χ^2_2 = 9.32, P = 0.009), a significant decrease of HEX activity after heat stress (χ^2_1 = 4.46, P = 0.03), and a non-significant interaction between thermal selection and thermal exposure (χ^2_2 = 2.28, P = 0.32). Looking for selection effects on each exposure treatment (Fig. 1A), we found that HEX basal activity evolved to lower values in the slow-ramping selected lines (Tukey P-adjusted value = 0.02), whereas fast-ramping selected lines showed similar HEX basal activity than control lines (Tukey P-adjusted value = 0.35). On the other hand, HEX induced activity was not different between thermal selection lines (Tukey P-adjusted value > 0.5). For PGI activity (Fig. 1B), we did not find significant effects of thermal selection (χ^2_2 = 3.43, P = 0.18), thermal exposure (χ^2_1 = 0.42, P = 0.52), neither significant interaction between these factors (χ^2_2 = 2.10, P = 0.35). For PGM activity (Fig. 1C), we did not find significant effect of the thermal selection regimens (χ^2_2 = 0.70, P = 0.71), thermal exposure (χ^2_1 = 1.42, P = 0.23), neither significant interaction between these factors (χ^2_2 = 1.16, P = 0.56). Finally, G6PD activity was significatively different between the thermal selection regimens (χ^2_2

= 6.7, P = 0.04; Fig. 1D). On the other hand, G6PD activity did not show significant differences between thermal treatments (χ^2_1 = 1.35, P = 0.25) neither a significant interaction between thermal selection and thermal exposure (χ^2_2 = 1.78, P = 0.41). Looking for selection effects on each exposure treatment, probably because differences between thermal selection regimens were close to significance, we did not find *a posteriori* significant differences between thermal selection regimens within each thermal stress treatment (Tukey P-adjusted value ≥ 0.1).

Regarding to the variability present among replicated lines, we found that the replicated lines showed a non-significant effect on the activity for all studied enzymes (χ^2_1 = 0, P = 1).

Early fecundity and egg-to-adult viability

Cumulative fecundity (Fig. 3) showed a significant effect of thermal selection (χ^2_2 = 17.83, P = 0.0001), oviposition day (χ^2_1 = 9248.33, P < 2.2 x 10^{-16}), and a significant interaction between both factors (χ^2_2 =30.51, P = 2.4 x 10^{-7}). Specifically, the cumulative fecundity in the control lines was lower than the slow-ramping selected lines (Tukey P-adjusted value = 0.02) and the fast-ramping selected (Tukey P-adjusted value = 0.04). Whereas both selected lines showed similar cumulative fecundity (Tukey P-adjusted value = 0.98). Looking for selection effects at each day (Fig. 3A), we observed that females of the selected lines lay more eggs than females of the control lines since day 3 after mating. Additionally, cumulative fecundity was not different between replicated lines (χ^2_1 = 2.03, *P* = 0.15).

For egg-to-adult viability (Fig. A), we found significant effects of thermal selection (χ^2_2 = 16.24, *P* = 0.0003, Fig. 4). Specifically, slow-ramping selected lines showed a significantly higher egg-to-adult viability than control lines (Tukey P-adjusted value = 0.0002), but not significantly different than fast-ramping selected lines (Bonferroni p-adjusted value = 0.07). On the other hand, fast-ramping

selected lines showed a similar egg-to-adult viability compared to control lines (Bonferroni p-adjusted value = 0.17). Finally, egg-to-adult viability were significantly different among replicated lines ($\chi^2_4 = 3.18$, $P = 1.4 \times 10^{-6}$), but this among-replicates variability did not blur the effects of thermal selection on the egg-to-adult viability.

Discussion

The mechanisms of tolerance to environmental stress are fundamental for the persistence of natural populations and biological diversity. Temperature is a conspicuous environmental factor that has influenced the evolution of metabolic and life-history traits in ectotherms, providing an interesting system to study the evolution and constraints of stress tolerance (Gilloly et al, 2001 Addo-Bediako et al, 2002; Brown et al, 2004; Padfield et al, 2016, Mallard et al, 2018). In this work, despite we did not find evidence of changes of metabolic rate in response to heat tolerance evolution in *D. subobscura*, we detected evolutionary correlated responses in a specific enzyme related to energy metabolism and positive correlated responses of fitness-related traits. Interestingly, these correlated responses of fitness-related traits to heat tolerance with no increase of the metabolic demand, which suggests that low-cost phenotypes are positively selected during heat tolerance challenges. Previously, our research team has been reported a correlated response of the locomotor performance to heat tolerance evolution in *D. subobscura*, corroborating that this species can adapt to increasing environmental temperatures (Mesas et al, 2021).

Despite the pervasive effects of temperature on metabolic traits, our findings show no evidence of a correlated response of RMR to heat tolerance evolution in *D. subobscura*. Different studies along geographical gradients have reported that organisms living in temperate environments have lower metabolic rates than their counterparts inhabiting cold habitats (Addo-Bediako et al, 2002; Sylvestre et al, 2007; Schaefer & Walters, 2010; Sinnatamby et al, 2015). In the same way, studies of selection

for stress resistance have found a reduction of metabolic rate in selected lines (Harshmann et al, 1999; Padfield et al, 2016). In addition, a study reported that populations of sticklebacks inhabiting geothermally warmed lakes showed a lower RMR than populations from cold lakes, but that this difference was only detected when both populations were acclimated to different temperatures (Pilakouta et al, 2020). However, our results agree a study published by Messamah et al (2017), who reported that the variation in the metabolic rate of 65 *Drosophila* species was not related to the annual mean temperature of their habitats of origin. Similarly, some studies in *D. melanogaster* show no differences in the metabolic rate between desiccation selected and control lines (Djawdan et al, 1997), and lines evolved in contrasting thermal condition (Alton et al, 2017). In this work, Alton et al (2017) suggest that the evolution of metabolic rate could depend on the interaction between temperature and resource availability, which would difficult the detection of metabolic response to thermal variability under typical maintaining conditions. In this way, our control and evolved populations were fed 'ad libitum' before measures, which could hide correlated responses of metabolic rate to heat tolerance evolution. Additionally, metabolic rate was measured at non-stressful conditions, which could have limited our capacity to detect differences between control and evolved lines.

Another possible explanation for not finding a correlated response of RMR to heat tolerance evolution is that metabolic rate is a complex trait that represents the total flux of energy at organismic level and that its evolutionary response could be constrained by the evolution of underlying traits such as metabolism-associated enzymes. For instance, Berrigan & Hoang (1999) reported a positive correlation between SMR and the activity of enzymes associated with energy metabolism across *Drosophila* genus. However, the moth *Amphion floridensis* showed that the oxygen consumption was no affected by changes in activity of glycolytic and mitochondrial enzymes,

suggesting that changes in enzyme activities associated with energy metabolism were not enough to modify the metabolic rate (Obrien & Suarez, 2001).

It is widely known that enzyme activity depends on the temperature: the lower the temperature, the lower the enzyme activity (Sokolova & Pörtner, 2001). Indeed, a study in *D. simulans* reports a reduction in the expression of genes related to glycolysis, Krebs cycle and oxidative phosphorylation in populations evolving in thermally fluctuating environments (Mallard et al, 2018). Similarly, we found that evolved lines showed a reduced activity in two out of four enzymes belong to the G6P branchpoint, but this correlated response to heat tolerance evolution depended on how thermal tolerance was selected: HEX evolved to lower activity in the slow-ramping selected lines, whereas analysis of G6PD detected significant differences between thermal selection regimens, but we cannot detect differences regarding to control lines. Interestingly, these correlated responses of HEX were only observed when flies were exposed to non-stressful conditions. Additionally, these same enzymes showed a reduced activity in flies exposed to 32 ºC in comparison to those exposed to non-stressful conditions (21 ºC), reveling a plastic response of some G6P branchpoint enzymes to temperature. These evolutionary and plastic responses of G6P branchpoint enzymes could be associated with changes in transcription rate of the enzyme coding genes as well as changes of thermostability and catalytic efficiency resulting from post-transcriptional modifications (Clark & Wang, 1994). However, we did not performed qPCR or RNA-seq approaches, which would be very useful to understand how heat tolerance evolution determine the specific or global patterns of gene expression in ectotherms.

Several studies have investigated the stress resistance evolution and have found correlated responses of metabolic enzymes of the G6P branchpoint and evolutionary responses in reproductive traits of insects (Kageyama & Ohnishi, 1971; Clark & Fucito, 1998; Harshman et al, 1999; Mallard et al, 2018), suggesting a correlated response between energy metabolism and fitness. In our study, we

observed a reduced activity of HEX in the slow-ramping selected lines, suggesting that individuals selected for high thermal tolerance also had lower metabolism (Santos et al, 2012). According to pace-to-life syndrome, individuals with fast metabolism should grow faster than their counterparts with slow metabolism, but at the cost to have shorter lifespan and lower fecundity (Stearns 1989; Polverino et al, 2018). In agreement with this idea, our results show that the evolution of heat tolerance is associated with a lower activity in the G6P branchpoint and higher fecundity. Our findings also agree to those reported by Talbert et al (2015) who found that *D. melanogaster* flies that express reduced HEX activity show longer life, whereas G6PD has been related to the reproduction and development of insects and fishes (Downer et al, 1985; Zera & Zhao, 2003b; Tripathi & Verma, 2004). For example, seasonal changes in G6PD activity have been associated with the reproductive cycle of catfish females, *Clarias batrachus* (Tripathi & Verma, 2004).

We also found a correlated response of the egg-to-adult viability to heat tolerance evolution, but only in the slow-ramping selected lines. Likely, evolution of fitness-related traits is associated with the pace-to-life syndrome as previously explained: the lower the activity of metabolic enzymes, the higher the fitness. However, this explanation is not completely valid for the fast-ramping selected lines, even though egg-to-adult viability is increased by 8% in these lines compared to control lines, and with some vials reaching up to 100% viability. However, enzyme activities have been related to development in *Drosophila* species: flies with short preadult development exhibit higher HEX and G6PD activities than flies with long development times (Marinkovic et al, 1986). Similar results were found in *D. simulans*, where populations evolved under different thermal regimens showed a low expression of genes coding for enzymes related to energy metabolism and also high fecundity (Mallard et al, 2018). Therefore, correlated responses of fitness-related traits suggest that evolutionary responses heat tolerance to global warming should be adaptive. However, these

findings need to be evaluated under realistic conditions to clearly understand the ecological and evolutionary implications of thermal evolution in natural populations.

In conclusion, we show that the evolution of tolerance to high temperatures does not involve a metabolic depression neither an increase in energy demand. Furthermore, increased heat tolerance evolution has positive consequences on fitness-related traits, including increased fecundity and preadult survival. As we previously showed (see Mesas et al, 2021), heat tolerance evolution induces correlated response on several traits, which finally result into an increased performance at high temperatures. Despite some evidence shows that thermal limits have limited evolutionary potential (Kellermann et al, 2012; Kelly et al, 2012), we have found through several works that upper thermal limits have genetic variation (Castañeda et al, 2019), which allows to *D. subobscura* populations adapt to local conditions (Castañeda et al, 2015) and respond to selection (Mesas et al, 2021). Thus, heat tolerance can evolve under different thermal scenarios but with different outcomes on related traits depending on the intensity of thermal stress, which change across spatial and temporal scales. Therefore, spatial and temporal variability of thermal stress intensity should be taking into account in future studies if we want to understand and predict the adaptive response to ongoing and future climatic conditions.

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Figure legends

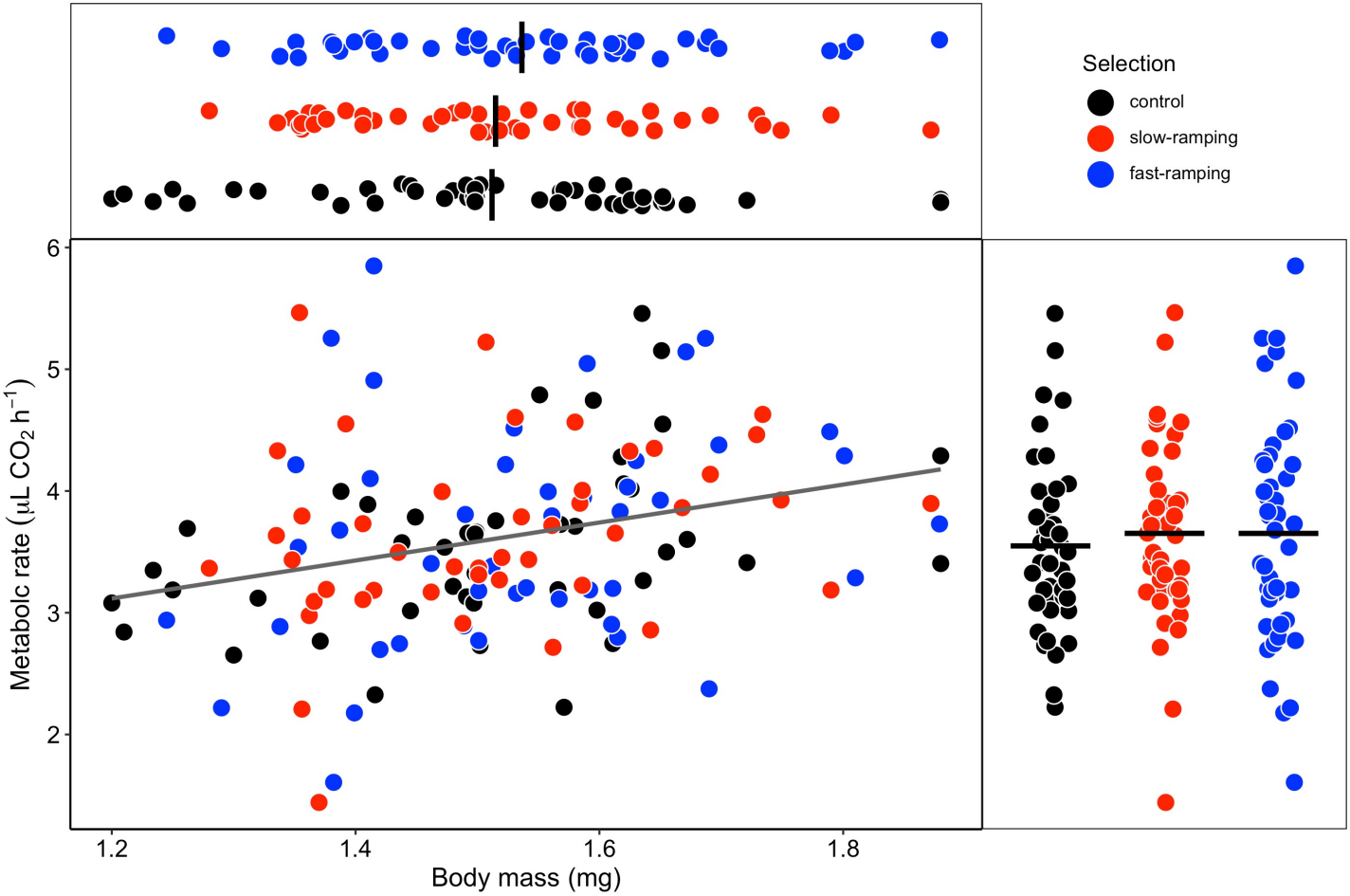
Figure 1. Relationship between body mass and metabolic rate (central panel) for flies of control lines (black circles), slow-ramping selected lines (red circles), and fast-ramping selected lines (blue circles) for increasing heat tolerance in *Drosophila subobscura*. The grey line represents the linear regression between both traits. Distribution of body mass and metabolic rate are showed in the right and upper panels, respectively, including the mean of each thermal selection regimen (black line). Non-significant different between thermal selection regimens were found for both traits (see text).

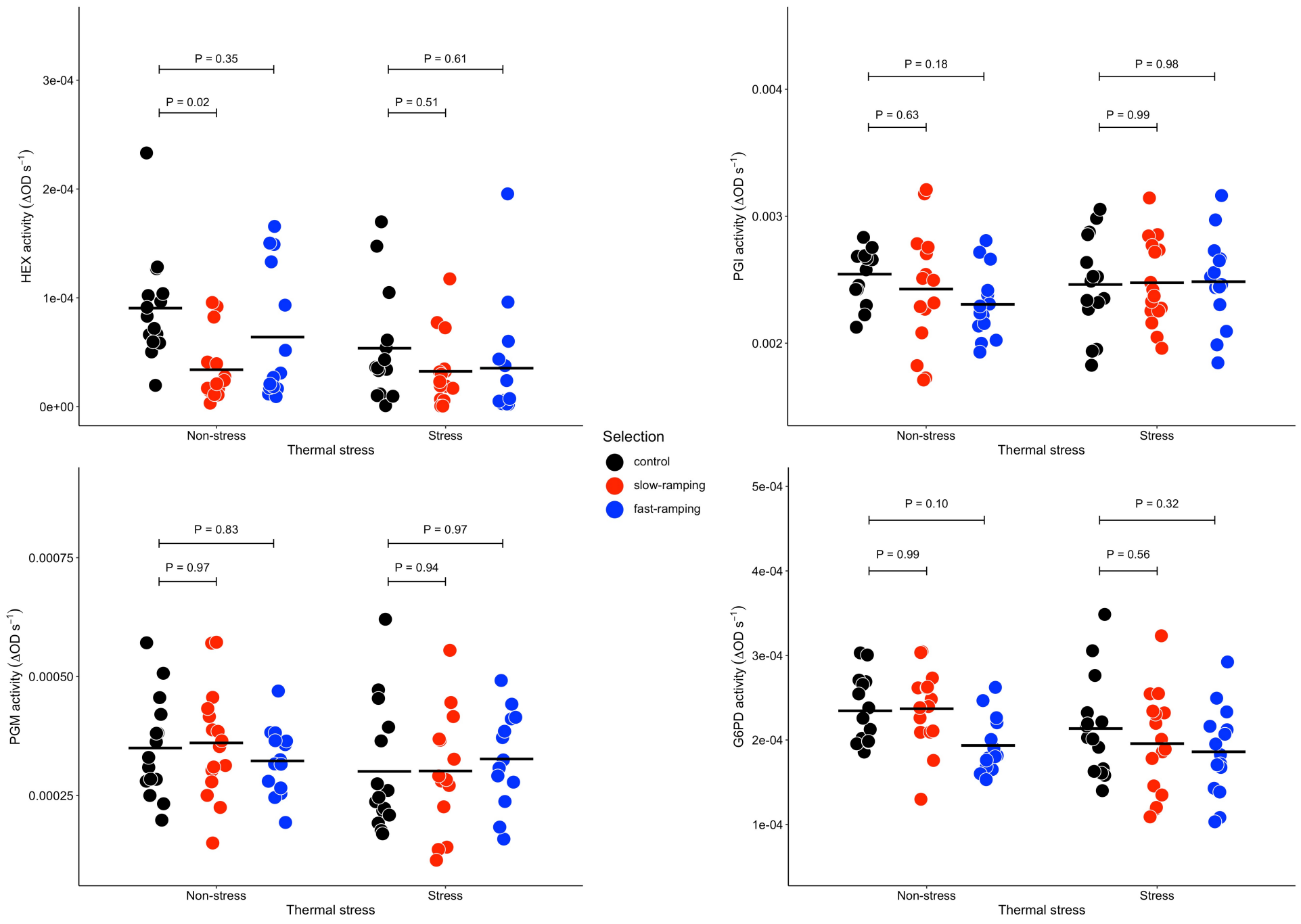
Figure 2. Activity of enzymes related to G6P branchpoint of flies of control lines (black circles), slow-ramping selected lines (red circles), and fast-ramping selected lines (blue circles) for increasing heat tolerance in *Drosophila subobscura* exposed to non-stressful (21 ºC) and stressful conditions (32 ºC): (A) hexokinase (HEX); (B) phophoglucomutase (PGM); (C) phosphoglucoisomerase (PGI); and (D) glucose-6-phosphate dehydrogenase (G6PD). Horizontal lines show the mean for each group. P-values above whiskers show the results of *a posteriori* comparisons between control and selected lines using Tukey tests.

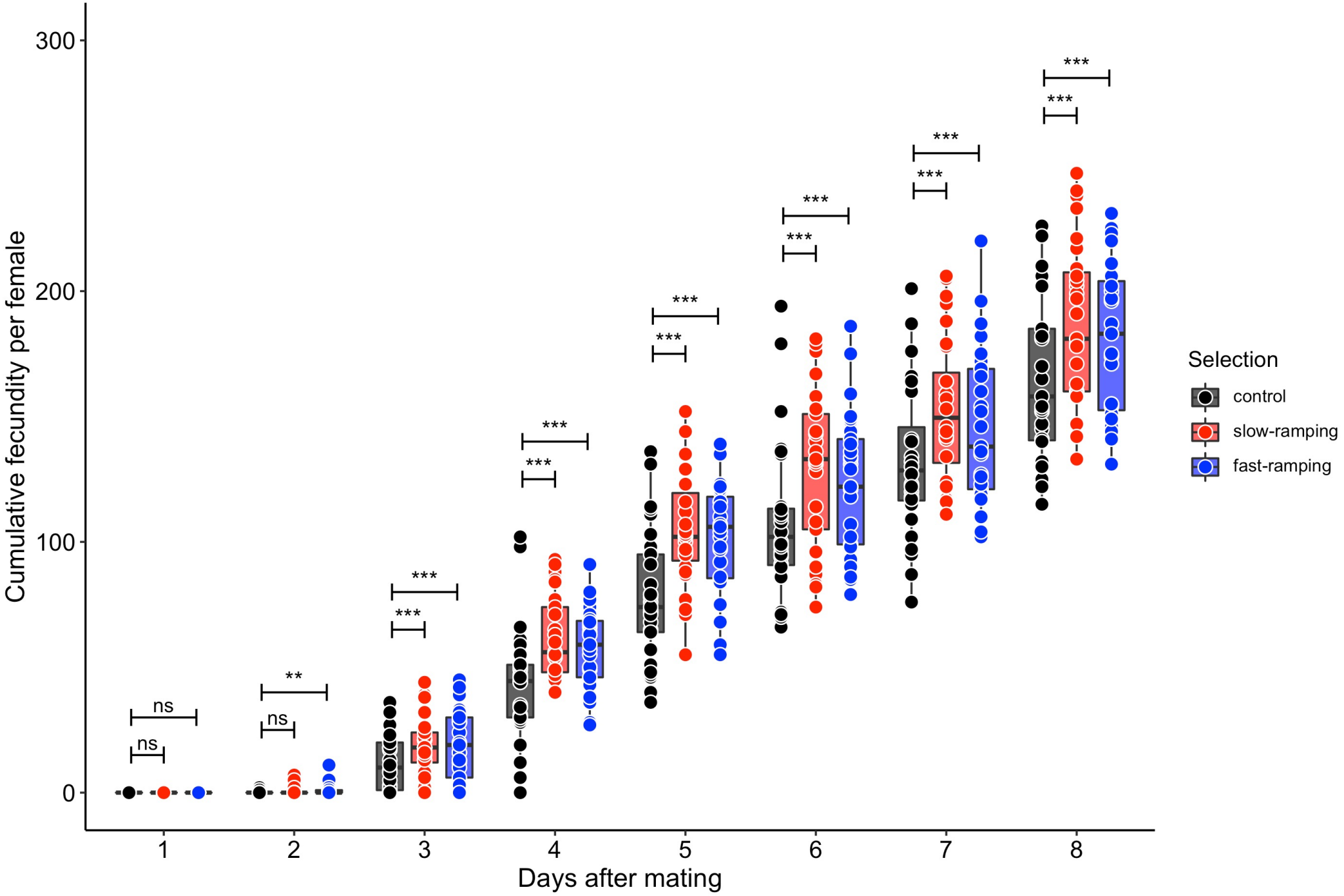
Figure 3. Cumulative fecundity along first 8 days after mating for females of control lines (black circles and boxplots), slow-ramping selected lines (red circles and boxplots), and fast-ramping selected lines (blue circles and boxplots) for increasing heat tolerance in *Drosophila subobscura*. Boxplots show median and the interquartile range (IRQ) and vertical whiskers represent the

1.5*IQR. P-values above horizontal whiskers show the results of *a posteriori* comparisons between control and selected lines using Tukey tests (ns: $P > 0.05$, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

Figure 4. Egg-to-adult viability of flies of control lines (black circles), slow-ramping selected lines (red circles), and fast-ramping selected lines (blue circles) for increasing heat tolerance in *Drosophila subobscura*. Horizontal lines show the mean for each group. P-values above horizontal whiskers show the results of *a posteriori* comparisons between control and selected lines using Tukey tests.


Selection
control
slow-ramping
fast-ramping
Metabolic rate (μL CO2 h−1)
6
5
4
3
2
1.2
1.4
1.6
1.8
Body mass (mg)


HEX activity (ΔOD s^{-1})
3e-04
2e-04
1e-04
0e+00
P = 0.35
P = 0.02
P = 0.61
P = 0.51
Non-stress
Stress
Thermal stress
PGI activity (ΔOD s^{-1})
0.004
0.003
0.002
P = 0.18
P = 0.63
P = 0.98
P = 0.99
Non-stress
Stress
Thermal stress
Selection
control
slow-ramping
fast-ramping
PGM activity (ΔOD s^{-1})
0.00075
0.00050
0.00025
P = 0.83
P = 0.97
P = 0.97
P = 0.94
Non-stress
Stress
Thermal stress
G6PD activity (ΔOD s^{-1})
5e-04
4e-04
3e-04
2e-04
1e-04
P = 0.10
P = 0.99
P = 0.32
P = 0.56
Non-stress
Stress
Thermal stress


Cumulative fecundity per female
300
200
100
0
1
2
3
4
5
6
7
8
Days after mating
ns
ns
**
ns
***
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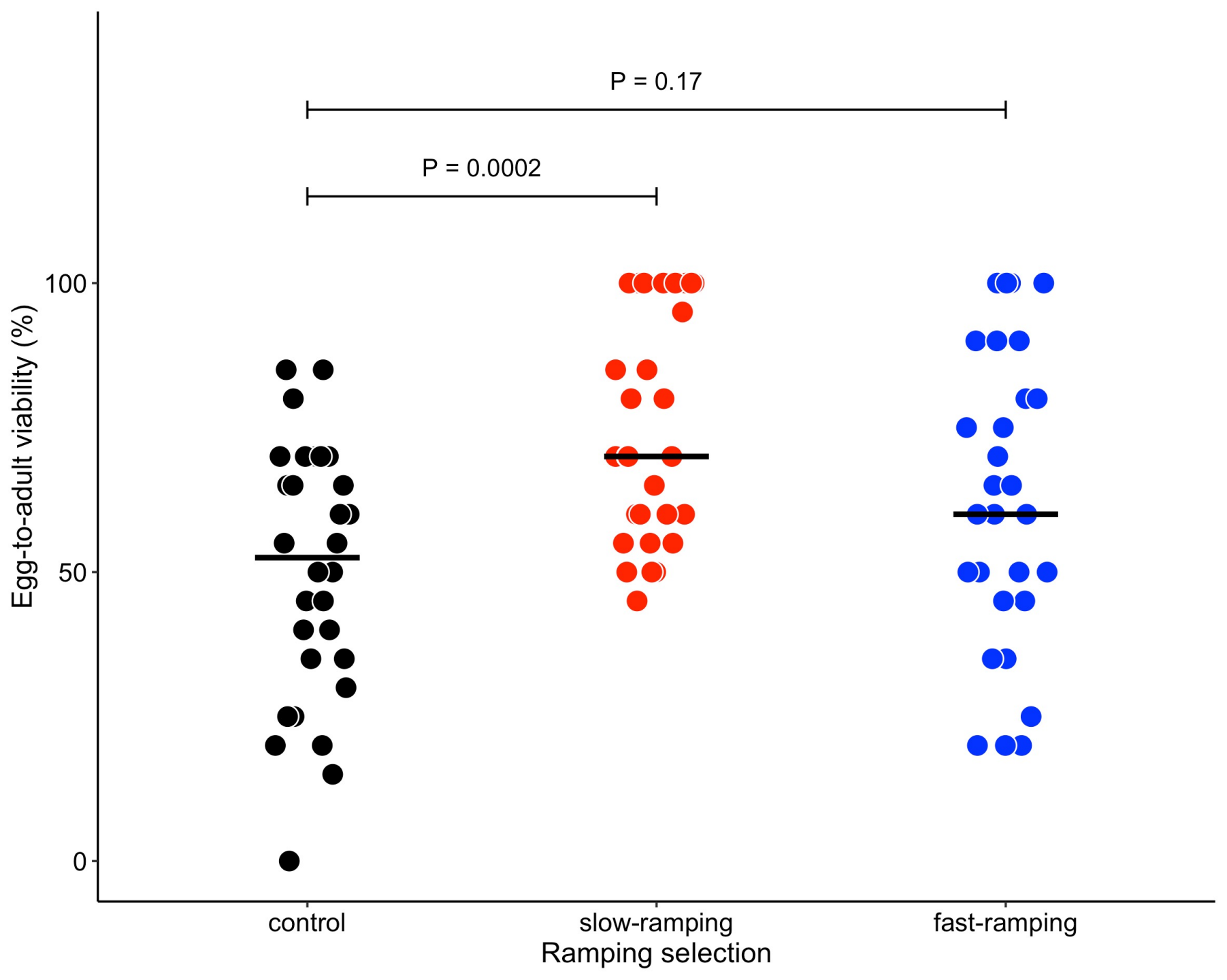
Selection
control
slow-ramping
fast-ramping


P = 0.17
P = 0.0002
100
50
0
Egg-to-adult viability (%)
control
slow-ramping
fast-ramping
Ramping selection